Resveratrol attenuates skeletal muscle atrophy induced by chronic kidney disease via MuRF1 signaling pathway

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Abstract

Skeletal muscle atrophy is an important clinical characteristic of chronic kidney disease (CKD); however, at present, the therapeutic approaches to muscle atrophy induced by CKD are still at an early stage of development. Resveratrol is used to attenuate muscle atrophy in other experimental models, but the effects on a CKD model are largely unknown. Here, we showed that resveratrol prevented an increase in MuRF1 expression and attenuated muscle atrophy in vivo model of CKD. We also found that phosphorylation of NF- κ B was inhibited at the same time. Dexamethasone-induced MuRF1 upregulation was significantly attenuated in C2C12 myotubes by resveratrol in vitro, but this effect on C2C12 myotubes was abrogated by a knockdown of NF- κ B, suggesting that the beneficial effect of resveratrol was NF- κ B dependent. Our findings provide novel information about the ability of resveratrol to prevent or treat muscle atrophy induced by CKD.

Keywords: Resveratrol; Chronic kidney disease; Muscle atrophy; MuRF1; Signaling pathway

1. Introduction

Muscle wasting is a common clinical characteristic of patients with chronic kidney disease (CKD) and increases morbidity and the risk of death [1]. Skeletal muscle atrophy is an effective indicator of muscle wasting, which is defined as a decrease in the mass of skeletal muscle. Molecular pathways underlying muscle atrophy are complicated, and many studies have shown that multiple pathways lead to skeletal muscle atrophy in patients with CKD [2]. Muscle ring-finger 1 (MuRF1) consists of 353 amino acid residues and contains a canonical N-terminal RING domain, which has been identified as a key muscle-specific E3 ubiquitin ligase that is highly expressed during muscle atrophy in CKD and increases muscle proteolysis by the ubiquitin-proteasome system (UPS) [3-4]. The expression of MuRF1 is regulated by NF- κ B during muscle atrophy, as revealed in MuRF1^{-/-} mice: a significant reduction in muscle loss in these mice revealed that transcriptional activation of MuRF1 by NF- κ B is a key step in NF- κ B-induced muscle atrophy [5].

Current strategies aimed at preventing muscle atrophy in clinical practice are focused on correction of acidosis plus promotion of physical exercise among patients with CKD, whereas in CKD mice with muscle atrophy, low-frequency electrical stimulation has been found to mimic acupuncture or exercise, both of which significantly improve weights of the soleus and extensor digitorum longus (EDL) muscles [6]. Currently, therapeutic approaches to muscle atrophy in CKD are still at early stages of development [7]. Specific therapies that inhibit a relevant signal transducer need to be developed to reduce the skeletal muscle atrophy induced by CKD.

Resveratrol (3,5,4'-trihydroxystilbene) is a natural polyphenol present in peanuts, pines, the skin of grapes, and red wine [8]. It has been shown to have many beneficial biological effects, including cardioprotection, antioxidant effects, inhibition of NF- κ B activity, and activation of AMP-activated protein kinase (AMPK) [9-11]. In addition, resveratrol has been reported to stimulate activity of histone deacetylase SIRT1; this effect may represent a key mechanism of action of this drug [12].

Growing evidence indicates that resveratrol may have beneficial effects in various muscle atrophic conditions, such as diabetes, cancer cachexia, and Duchenne muscular dystrophy; it can attenuate skeletal muscle atrophy by multiple mechanisms [13-15].

A recent study showed that resveratrol may be a promising therapy for CKD patients [16], although studies evaluating its effects in CKD are scarce. Some clinical studies based on polyphenol-containing supplementation have shown anti-inflammatory effects and improvements in antioxidant activity in patients with CKD or end stage renal disease (ESRD) [17-18]. During basic research on CKD, Liang et al. found that resveratrol treatment can inhibit oxidative stress and renal interstitial fibrosis[19]. Until recently, however, no study has provided comprehensive insights into the effects of resveratrol on CKD-induced skeletal muscle atrophy although it is plausible that resveratrol can provide several benefits that may attenuate muscle atrophy. The protective effect of resveratrol against skeletal muscle atrophy during CKD and the effect on NF- κ B activation are not well understood. In the present study, we tested the hypothesis that resveratrol prevents CKD-induced muscle atrophy by inhibiting the expression of MuRF1 and protein degradation, and we determined whether the protective effects of resveratrol are NF- κ B dependent.

2. Materials and methods

2.1. Mouse strains and the CKD model

C57BL/6 mice aged 8 weeks were randomly subdivided into two groups (Sham group and CKD group); 5/6 nephrectomy (Nx) was utilized to create the CKD model and means resection of approximately 2/3 of the left kidney, followed by removal of the right kidney 1 week later. Sham-treated mice received sham operations; the appropriate kidney was exposed and mobilized but not treated in any other way.

After the CKD model was established, the mice were randomly redistributed into four groups, namely, Sham treatment + vehicle (n = 5), Sham treatment + gavage with 200 mg/(kg·day) resveratrol (n = 5), CKD + vehicle (n = 5), CKD + gavage with 200 mg/(kg·day) resveratrol (n = 5); the duration of resveratrol treatment was 21 days.

Vehicle-treated groups received an equal volume of normal saline. Resveratrol was purchased from Copolyton Chemical Materials Co., Ltd., Shanghai, China. The use of animals in our studies was in compliance with protocols approved by the Institutional Animal Care and Use Committee of Xinhua Hospital.

The levels of blood urea nitrogen (BUN) and creatinine in mice were measured using Infinity™ Urea (Nitrogen) and Liquid Stable Reagent (Thermo Fisher Scientific).

2.2. Isolation of total RNA and quantitative real-time PCR (RT-qPCR)

TRIzol reagent (Sigma-Aldrich, St. Louis, MO) was used to extract total RNA from tissue samples. Reverse transcription reactions were performed using the iScript cDNA Synthesis Kit (Quanta, Gaithersburg, MD). SYBR Green Real-Time Quantitative PCR was performed on a Bio-Rad CFX96 System (Bio-Rad Laboratories). The amplification conditions were as follows: 3 min at 95°C and 40 cycles of 15 s at 95°C, 60 s at 60°C, and 5 min at 95°C. The expression levels of all mRNAs were normalized to GAPDH. The primer sequences for RT-qPCR were as follows: MuRF1 Forward: 5'-AGTGTCCATGTCTGGAGGTCGTTT-3', Reverse: 5'-ACTGGAGCACTCCTGCTTGCTAGAT-3'; GAPDH Forward: 5'-ACCACCATGGAGAAGGCCGG-3', Reverse: 5'-CTCAGTGTAGCCCAAGATGC-3'; NF-κB Forward: 5'-AGTTTGACGGTGAGCTGGTA-3', Reverse: 5'-GCCTCGGCCTGCCGCAAGCCT-3'.

2.3. Protein extraction, western blotting, and antibodies

Total protein extracts were prepared as follows; for western blotting, muscles were lysed in RIPA buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.025% SDS, 1 mM sodium orthovanadate, 10 mM NaF, 25 mM β-glycerophosphate) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). After centrifuging at 12,000 × g for 12 min at 4°C, the supernatants were subjected to western blotting. Nuclear protein extracts were prepared using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Asheville, NC). The following antibodies were used: anti-phospho-NF-κB p65 (S536) antibody (1:500, Cell Signaling Technology),

anti-NF- κ B p65 (1:1000, Cell Signaling Technology), anti-I κ B- α (1:1000, Cell Signaling Technology), anti-GAPDH (1:1000, Cell Signaling Technology), and an anti-MuRF1 antibody (1:1000, ECM Biosciences). The data on the target protein expression were normalized to GAPDH.

2.4. Protein synthesis and degradation using an isotopic technique

To measure protein synthesis and degradation in the muscles of CKD mice, we incubated soleus and EDL muscles in 3 mL of Krebs–Henseleit bicarbonate buffer containing 0.5 mmol/L L-phenylalanine, 10 mmol/L glucose, and 0.05 μ Ci of L- 14 C-phenylalanine (MP Biomedicals, Solon, OH) for 30 min. After gassing with 95% O₂/5% CO₂, the muscles were incubated for 30 min. We incubated the muscles in the fresh buffer gassed with 95% O₂/5% CO₂ for another 2 h. The rate of protein synthesis was measured as incorporation of L- 14 C-phenylalanine into muscle protein. The rate of protein degradation was measured as the rate of release of tyrosine into the medium during the 2 h of incubation.

2.5. Immunostaining and measurement of the size of myofibers

The frozen TA muscles' slices (4 μ m thick) was fixed with 4% formaldehyde for 5 min, then blocked with a protein blocking solution for 20 min. Dystrophin antibody (1:300, Abcam) was incubated with the slides at 4°C overnight. The secondary Alexa Fluor-conjugated antibody (1:600, Life Technologies) was incubated with the muscles' slices for 30 min at room temperature. The areas of myofibers were measured using the NIS-Elements software (Nikon, USA), and at least 1000 myofibers per TA muscle were analyzed.

2.6. Cell culture and transfection with NF- κ B small interfering RNA (siRNA)

C2C12 mouse myoblasts (ATCC; Manassas, VA) were routinely cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and 1% of the penicillin/streptomycin solution (Haoran Biological Technological Co., Shanghai,). At 90% confluence, FBS was replaced with 2% horse serum to induce C2C12 myoblast cells to differentiate into myotubes. The C2C12 myotubes were transfected with scramble control siRNA

(siCTL) or NF- κ B siRNA (GenePharma Co., Shanghai) for 48 h in DMEM containing 2% of horse serum in 6-well plates. After 48 h, the myotubes were treated with 1 μ M dexamethasone (Dex), 100 μ M resveratrol, co-administration of Dex and resveratrol for 24 h. We used Lipofectamine RNAiMAX Reagent (Invitrogen) to transfect the siRNA. The working concentrations of Dex and resveratrol were based on methods reported in other studies [20-21].

2.7. Statistical analyses

Data are presented as mean $\pm$ standard error of the mean (SEM) of three biological replicates. The body weight results were subjected to one-way ANOVA. Other results were statistically analyzed by two-tailed Student's *t* test to determine *p* values. Differences were considered statistically significant at *p* < 0.05.

3. Results

3.1. Skeletal muscle atrophy is a characteristic feature of the CKD model

To confirm this, body weight was measured once a week. We found that body weight loss was significant starting with week 3 and kept decreasing in CKD mice compared to the sham mice (Fig. 1A). As shown in Fig. 1B and C, there were significant increases in the serum levels of BUN and creatinine in CKD mice, indicating successful establishment of the CKD model. The ratio of tibialis anterior (TA) muscle weight to tibia length was significantly lower in CKD mice compared to the sham mice (Fig. 1D). Using qPCR, we detected a significant increase in transcript levels of MuRF1 in CKD mice (Fig. 1E). At the same time, the protein levels of MuRF1 were higher in CKD mice than in sham mice (Fig. 1F).

3.2. Resveratrol attenuates skeletal muscle atrophy in CKD mice

To test whether the beneficial effects of resveratrol can alleviate muscle atrophy in CKD, first, we examined the body weight of mice in each group. CKD mice were given resveratrol (200 mg/[kg·day] by gavage) for 3 weeks, and we observed a significant increase in the body weight of CKD mice compared to pair-fed mice that did not receive resveratrol. CKD mice receiving resveratrol showed the mean weight gain of 34.7% $\pm$ 7.7% relative to their baseline weights during the experimental

period. Nonetheless, we did not find any difference between the sham mice treated and not treated with resveratrol although all the groups had equivalent food intakes (data not shown). Concomitantly with the increase in body weight, an improvement in the ratio of TA muscle weight to tibia length was detected in CKD mice treated with resveratrol (Fig. 2A). We measured the distribution of the myofiber cross-sectional area in TA muscles from sham mice and CKD mice treated or not treated with resveratrol: an increase in muscle mass in CKD mice treated with resveratrol was found, and a rightward shift in the distribution of myofiber sizes was also observed (Fig. 2B). The grip strength was significantly improved in CKD mice treated with resveratrol (Fig. 2C). These findings revealed that resveratrol at the dose of 200 mg/(kg-day) can protect against CKD-induced muscle atrophy, but this dose did not cause an increase in muscle mass of sham mice, suggesting that the protective effects of resveratrol may not be due to a simple increase in muscle mass.

3.3. Resveratrol improves muscle protein metabolism associated with suppression of NF- κ B activity in CKD mice

To identify the mechanism by which resveratrol influences muscle atrophy, we analyzed protein synthesis and degradation in muscles of CKD mice treated or not treated with resveratrol. A significant increase in muscle protein synthesis and a significant decrease in protein degradation were observed in CKD mice treated with resveratrol as compared with pair-fed CKD mice without resveratrol (Fig. 3A and B). In accordance with the results of measurement of protein synthesis and degradation, we then focused on the changes in the muscle atrophy-related protein: the mRNA levels of MuRF1 in CKD mice treated with resveratrol were suppressed by resveratrol, i.e., MuRF1 mRNA levels decreased to 50% of baseline (Fig. 3C). Using immunoblots, we confirmed that the protein expression of MuRF1 in CKD mice was suppressed by resveratrol (Fig. 3D), these results indicate that resveratrol may alleviate muscle atrophy in CKD mice.

CKD induced significant phosphorylation of NF- κ B p65, but this effect was markedly attenuated by resveratrol. In addition, as shown in Fig. 3E, resveratrol inhibited the CKD-induced degradation of I κ B- α . These data suggest that the

beneficial effect of resveratrol on CKD mice may be associated with suppression of the NF- κ B signaling pathway.

3.4. Via inhibition of activation of NF- κ B in C2C12 myotubes, resveratrol blocks MuRF1 expression induced by Dex stimulation

To further ascertain the effect of resveratrol on the regulation of muscle atrophy, we examined how resveratrol changes the expression of MuRF1 in C2C12 myotubes. Because Dex was reported to increase MuRF1 expression in muscle cells [22], we exposed C2C12 myotubes to 1 μ M Dex for 24 h and saw a significant increase in MuRF1 expression. When C2C12 myotubes were simultaneously treated with resveratrol, using RT-qPCR, we found that MuRF1 and NF- κ B mRNA upregulation was attenuated (Fig. 4A). We tested the efficiency of the knockdown of NF- κ B and found that transfection of NF- κ B siRNA downregulated NF- κ B at the transcriptional level by 83% in C2C12 myotubes (Fig. 4B). In C2C12 myotubes transfected with NF- κ B siRNA, we did not detect a difference in mRNA expression of MuRF1 between groups Dex and coadministration of Dex and resveratrol (Fig. 4C). As shown in Fig. 4D, we confirmed that the protein levels of MuRF1 were changed in accordance with the results of mRNA level. Taken together, these results indicate that via inhibition of NF- κ B in C2C12 myotubes, resveratrol can attenuate upregulation of MuRF1 by Dex.

4. Discussion

Our results suggest that resveratrol can inhibit CKD-induced skeletal muscle atrophy and downregulate MuRF1, and that these effects of resveratrol are at least in part mediated by NF- κ B. In addition, this study suggests that a resveratrol dose of 200 mg/(kg·day) may be necessary to prevent CKD-induced muscle atrophy in animal models. We can hypothesize that this treatment can be used in clinical practice, where a reduction in CKD-induced muscle atrophy may benefit a large number of patients.

Skeletal muscle atrophy in CKD patients presents as muscle loss, often associated with anorexia, inflammation, and insulin resistance, while the metabolism of muscle protein in CKD is affected mainly by increased protein degradation, rather

than by reduced protein synthesis[23]. The precise role of the complex signaling mechanism involved in muscle atrophy has not been completely delineated in CKD to date. Lately, some evidence showed that increased UCP3 expression may be important for skeletal muscle atrophy in CKD [24], suggesting that inhibition of UCP3 expression may play a role in the muscle atrophy. As an important UCP3 component, MuRF1 performs a special function in skeletal muscle atrophy in vivo [25].

NF- κ B is involved in many cellular processes, such as immune and inflammatory responses, developmental processes, cell growth, and apoptosis [26]. One study showed that NF- κ B may be involved in medical conditions associated with muscle wasting [27]. The NF- κ B pathway activation was also mentioned as one of the main signaling cascades regulating the expression of muscle-specific E3 [28]. Our research yielded the same result as the above study did: MuRF1 expression was increased by NF- κ B activation in a CKD model.

It should be noted that the potential mechanisms underlying the anticatabolic effects of resveratrol have been described elsewhere, including antioxidant effects, inhibition of NF- κ B activities, prevention of insulin resistance, and increased microvascular recruitment in skeletal muscle; several of these mechanisms are interconnected [29]. Many studies have revealed that different effects of resveratrol may be regulated by different mechanisms in various muscle atrophy models. For example, Shadfar found that resveratrol inhibits tumor-enhanced cardiac atrophy in vivo possibly through inhibition of NF- κ B and MuRF1 activities [14]. Wyke reported that resveratrol significantly attenuates weight loss and protein degradation in skeletal muscle and significantly reduces NF- κ B DNA-binding activity in cancer cachexia [30]. Alamdari reported that the protective effects of resveratrol against dexamethasone-induced MuRF1 expression are at least SIRT1-dependent in cultured L6 myotubes [31]. Nevertheless, some studies have shown that MuRF1 is regulated by other transcription factors, such as the FOXO family; therefore, it is possible that resveratrol downregulates MuRF1 via a mechanism other than the NF- κ B pathway [32]. Although these results suggest that resveratrol targets a different mechanism in a

different muscle atrophy model, these data may also suggest that resveratrol inhibits the expression of MuRF1 and UCP3 through different mechanisms.

Until now, the effects of resveratrol on CKD-induced muscle atrophy have not been reported. In our CKD model, we confirmed that the beneficial effect of resveratrol is in part mediated by attenuated degradation of I κ B- α and by inhibited activation of NF- κ B and reflects inhibited upregulation of the ubiquitin ligase MuRF1, thus attenuating muscle atrophy. We also found that resveratrol attenuates protein degradation instead of enhancing protein synthesis *in vivo*. The protective effects of resveratrol against Dex-induced MuRF1 upregulation were almost abrogated in C2C12 myotubes transfected with NF- κ B siRNA, further suggesting that this effect of resveratrol was in part mediated by the NF- κ B signaling pathway.

In our study, resveratrol prevented an increase in MuRF1 expression in cultured C2C12 cells and in the CKD model, we obtained evidence that this effect of resveratrol is NF- κ B dependent. This study for the first time shows that resveratrol therapy may be effective against CKD-induced atrophy through the inhibition of NF- κ B activity in muscle, further supporting the potential prophylactic or therapeutic effects of resveratrol on muscle wasting. Therefore, expression of MuRF1 in CKD and the pathways involved in muscle atrophy processes require further discussion. Accordingly, the protective potential of resveratrol deserves further research in CKD models.

Conflict of interest

None of the authors have a conflict of interest with respect to this work.

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Figure Legends

Figure 1: Skeletal muscle atrophy is a specific feature of the CKD model

A: Body weight changes in sham-operated and CKD mice, * $p < 0.05$ vs sham-operated mice.

B: The serum level of BUN was significantly increased in CKD mice, * $p < 0.05$.

C: The serum level of creatinine was significantly increased in CKD mice in comparison with the sham group, * $p < 0.05$.

D: The ratio of TA muscle weight to tibia length was significantly lower in CKD mice compared to the sham group, * $p < 0.05$.

E: A significant increase in mRNA levels of MuRF1 was observed in CKD mice, * $p < 0.05$.

F: The protein levels of MuRF1 were higher in CKD mice than in the sham group, * $p < 0.05$.

Figure 2: Resveratrol attenuates skeletal muscle atrophy in CKD mice

A: The ratio of TA muscle weight to tibia length was increased in CKD mice treated with resveratrol relative to pair-fed mice that did not receive resveratrol. Res: resveratrol.

B: The myofiber cross-sectional area in TA muscles from the sham group and CKD mice treated or not treated with resveratrol. A rightward shift in the distribution of myofiber sizes was detected in CKD mice treated with resveratrol.

C: The grip strength was significantly improved in CKD mice treated with resveratrol.

Figure 3: Resveratrol improves muscle protein metabolism associated with suppression of NF- κ B activity in CKD mice

A: Protein synthesis was measured with ^{14}C -Phe incorporation in the soleus and EDL muscles from CKD mice treated with vehicle or resveratrol.

B: The rates of protein degradation were measured with the tyrosine release in the soleus and EDL muscles from CKD mice treated with vehicle or resveratrol.

C: Resveratrol suppressed the mRNA level of MuRF1 in CKD mice by RT- qPCR.

D: Immunoblots show that resveratrol inhibited the protein expression of MuRF1 in CKD mice.

E: Immunoblots show that resveratrol suppressed NF- κ B p65 phosphorylation and I κ B- α degradation in CKD mice.

Figure 4: Resveratrol blocks MuRF1 upregulation induced by Dex stimulation via inhibiting the activation of NF- κ B in C2C12 myotubes

A: C2C12 myotubes were simultaneously treated with Dex and resveratrol, MuRF1 and NF- κ B mRNA levels were decreased.

B: C2C12 myotubes were transfected with scramble control siRNA(siCTL) or NF- κ B siRNA for 48 h, and the knockdown efficiency was confirmed by RT-qPCR.

C: In C2C12 myotubes transfected with NF- κ B siRNA or siCTL, the changes in MuRF1 mRNA under the influence of Dex or coadministration with resveratrol were confirmed by RT-PCR.

D: Immunoblots show changing trends of MuRF1 protein expression under different conditions in C2C12 myotubes.

A **B** **C**